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The Induction of Seed Germination in *Arabidopsis thaliana* Is Regulated Principally by Phytochrome B and Secondarily by Phytochrome A[1]

Tomoko Shinomura, Akira Nagatani, Joanne Chory, and Masaki Furuya*

Advanced Research Laboratory, Hitachi Ltd., Hatoyama, Saitama, Japan 350-03 (T.S., M.F.); Laboratory of Photoperception and Signal Transduction, Frontier Research Program, RIKEN Institute, Wako, Saitama, Japan 351-01 (A.N.); and Plant Biology Laboratory, The Salk Institute, San Diego, California 92186 (J.C.)

We examined whether spectrally active phytochrome A (PhyA) and phytochrome B (PhyB) play specific roles in the induction of seed germination in *Arabidopsis thaliana* (L.) Heynh., using PhyA- and PhyB-null mutants, *fre1-1* (A. Nagatani, J.W. Reed, J. Chory [1993] Plant Physiol 102: 269-277) and *hy3*-Bo64 (J. Reed, P. Nagpal, D.S. Poole, M. Furuya, J. Chory [1993] Plant Cell 5: 147-157). When dormant seeds of each genotype imbibed in the dark on aqueous agar plates, the *hy3* (*phyB*) mutant did not germinate, whereas the *fre1* (*phyA*) mutant germinated at a rate of 50 to 60%, and the wild type (WT) germinated at a rate of 60 to 70%. By contrast, seeds of all genotypes germinated to nearly 100% when plated in continuous irradiation with white or red light. When plated in continuous far-red light, however, frequencies of seed germination of the WT and the *fre1* and *hy3* mutants averaged 14, nearly 0, and 47%, respectively, suggesting that PhyB in the red-absorbing form prevents PhyA-dependent germination under continuous far-red light. When irradiated briefly with red or far-red light after imbibition for 1 h, a typical photoreversible effect on seed germination was observed in the *fre1* mutant and the WT but not in the *hy3* mutant. In contrast, when allowed to imbibe in the dark for 24 to 48 h and exposed to red light, the seed germination frequencies of the *hy3* mutant were more than 40%. Immunoblot analyses of the mutant seeds showed that PhyB apoprotein accumulated in dormant seeds of the WT and the *fre1* mutant as much as in the seeds that had imbibed. In contrast, PhyA apoprotein, although detected in etiolated seedlings grown in the dark for 5 d, was not detectable in the dormant seeds of the WT and the *hy3* mutant. The above physiological and immunochemical evidence indicates that PhyB in the far-red-absorbing form was stored in the *Arabidopsis* seeds and resulted in germination in the dark. Hence, PhyA does not play any role in dark germination but induces germination under continuous irradiation with far-red light. Finally, we examined seeds from a signal transduction mutant, *det1*, and a *det1*/*hy3* double mutant. The *det1* seeds exhibited photoreversible responses of germination on aqueous agar plates, and the *det1*/*hy3* double mutant seeds did not. Hence, DET1 is likely to act in a distinct pathway from PhyB in the photoregulation of seed germination.

In many wild plant species, seeds in a given population do not all germinate at the same time under a given set of conditions favorable for seedling growth (Frankland and Taylorson, 1983). When allowed to imbibe, some seeds can germinate in the dark, whereas others even in the same population fail to germinate and require some physical or chemical stimulus such as light (Borthwick et al., 1952), temperature (Roth-Begerano, et al., 1966; Mancinelli et al., 1967), and chemicals (Quatrano, 1987; Georghiou and Kendrick, 1991). Among these environmentally controlling stimuli, light is the best characterized factor. Borthwick et al. (1952) discovered the red/far-red photoreversible effect on seed germination in *Lactuca sativa*. Later, similar effects were found in *Lepidium* (Toole et al., 1955), tomato (Mancinelli et al., 1966), and many other species (Furuya, 1968; Frankland and Taylorson, 1983; Cone and Kendrick, 1986; Sage, 1992).

In the 1960s and 1970s, numerous data from physiological and spectrophotometrical studies revealed the existence of multiple molecular species of phytochrome-dependent responses (reviewed by Furuya, 1989, 1993). For instance, Borthwick et al. (1954) demonstrated that the rate of loss of red/far-red reversibility in the photoregulation of seed germination was slow and that at least 8 h was needed for 50% loss of photoreversibility. The germination frequency of partially or totally dark-germinating seeds was controlled by phytochrome, a portion of which was present as Pfr in seeds allowed to imibe in the dark (Mancinelli et al., 1967). These observations were not consistent with in vivo spectrophotometrical reports that the disappearance of Pfr always occurred in etiolated tissues with a half-life of approximately 60 to 90 min at physiological temperature (Butler et al., 1963; Furuya and Hillman, 1964). In the 1980s, however, it became clear that phytochrome consisted of a family of photoreceptors, differing in amino acid sequences in their apoproteins (PHY) (Abe et al., 1989). The phytochromes were classified into two groups (type I labile and type II stable phytochromes) on the basis of stability of the Pfr form (Furuya, 1989). Based on this and other results, Smith et al. (1991) suggested that the phytochrome playing a major role in the photoregulation of seed germination might be a stable phytochrome.

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* Corresponding author; fax 81-492-96-7511.

Abbreviations: EOD-FR, end-of-day far-red light; Phy, spectrally active phytochrome; PHY, apoprotein of Phy; *PHY*, gene of wild-type PHY; *phy*, gene of mutant PHY; $PhyB_{fr}$, PhyB in the far-red-absorbing form; $PhyB_r$, PhyB in the red-absorbing form; WT, wild type.

In *Arabidopsis thaliana*, three distinct phytochrome genes, *PHYA*, *PHYB*, and *PHYC*, were isolated and sequenced, and amino acid identities among different members of the family were found to be as low as 50% (Sharrock and Quail, 1989). The existence of multiple species of Phy, together with the diversity of Phy responses and response modes, led to the hypothesis that different molecular species of Phy may have discrete functions (Furuya, 1993). Current understanding of Phy function has been greatly advanced through the analysis of transgenic plants that overexpress PHYA or PHYB (Kay et al., 1989; Keller et al., 1989; Boylan and Quail, 1991; Nagatani et al., 1991b; Wagner et al., 1991) and mutants that are deficient in one of the molecular species of Phy(s). For examples, the *hy8* mutant (Parks and Quail, 1993), the *fre1* mutant (Nagatani et al., 1993), and the *fhy2* mutant (Whitelam et al., 1993) of *Arabidopsis* lack PHYA, and the *hy3* mutant of *Arabidopsis* (Nagatani et al., 1991a; Somers et al., 1991; Reed et al., 1993), the *lh* mutant of cucumber (López-Juez et al., 1992), and the *ein* mutant of *Brassica* (Devlin et al., 1992) lack PHYB. At present, the *hy3* mutant of *Arabidopsis* (Koornneef et al., 1980) is the best-characterized mutant among a number of Phy-deficient mutants. The *HY3* locus encodes the *PHYB* gene, and the *hy3* mutants have mutations in the *PHYB* gene (Reed et al., 1993) and lack PHYB polypeptide (Nagatani et al., 1991a; Somers et al., 1991). The *FRE1* locus encodes *PHYA* because mutations in *fre1* indicate nonsense mutations in the *PHYA* genes (J.W. Reed, A. Nagatani, T.D. Elich, M. Fagan, and J. Chory, unpublished data) and PHYA was not detectable on immunoblots of the *fre1* mutant (Nagatani et al., 1993). These mutants are useful in studies designed to assign specific molecular species of Phy for known Phy effects, including the photoresponse of seed germination.

In *A. thaliana*, the action spectra for photoreversible induction and inactivation of seed germination was determined and indicated a phytochrome effect in the WT (Shropshire et al., 1961). Moreover, WT seed germination in the dark was specifically influenced by the spectral quality of the light regime under which the parent was grown, indicating that the seed population having the ability to germinate in the dark contains a favorably large amount of persistent phytochrome in the active form during the dormant period (McCullough and Shropshire, 1970). Later, fluence-response curves and action spectra for promotion and inhibition of seed germination were measured in WT and long-hypocotyl mutants of *Arabidopsis* (Cone and Kendrick, 1985). In the present study, we examined Phy-induced seed germination in WT and *hy3* and *fre1* mutants of *Arabidopsis* to assign specific molecular species of Phy for the photoresponse of seed germination.

An interesting question arises as to whether each Phy species has unique signal transduction pathways or whether they all share common pathways. To answer this question, studies using mutants of signal transduction pathways have been attempted (Chory, 1992). In the *det1* mutant of *Arabidopsis*, which constitutively displays many characteristics that are light dependent in WT (Chory et al., 1989), previous experiments have shown that the seeds germinate regardless of the presence or absence of light (Chory et al., 1989), and this also is seen in the *det1/hy3* double mutant (Chory, 1992). The *cop1* mutants of *Arabidopsis*, however, showed normal Phy control of seed germination (Deng et al., 1991). However, the control of seed germination is complex and is promoted not only by red-light irradiation but also by the presence of nitrate ions (Georghiou and Kendrick, 1991) and hormones (Quatrano, 1987) in the imbibition medium. Because early studies were performed on minimal salts plates containing trace elements, vitamins, and Suc, it was unclear whether the lack of light effect on *det1* and *det1/hy3* seed germination was due to supplements in the medium.

In the present study, we examined *det1* and *det1/hy3* seeds to determine whether they lose photoregulation of seed germination even if they are sown on aqueous agar medium. We report that PhyB principally controls seed germination in *A. thaliana*, PhyA plays only a supplementary role in the absence of PhyB, and the PhyB signaling pathway in germination may be different from the DET1 pathway using the *det1* and *det1/hy3* double mutants.

MATERIALS AND METHODS

Plant Materials

The alleles used in the present study were *fre1-1* (Nagatani et al., 1993), *hy3*(Bo64) (Koornneef et al., 1980), *hy3*(8–36) (Reed et al., 1993), *det1-1* (Chory et al., 1989), and *det1-1/hy3* (Bo64) (Chory, 1992) in *Arabidopsis thaliana* (L.) Heynh. The WT used in the present study was Landsberg *erecta*. The mutants used were either isolated in the Landsberg *erecta* background (*hy3*, *fre1*) or backcrossed into the Landsberg *erecta* background (*det1*, *det1/hy3*). The two *hy3* mutant alleles used, *hy3*(Bo64) and *hy3*(8–36), exhibited essentially the same responses in the present experiments, thus we show the data of *hy3*(Bo64) as representative of *hy3* mutants. Seeds of WT, *hy3*, *fre1*, *det1*, and *det1/hy3* were harvested from plants grown in temperature-controlled (23 ± 1°C) rooms under continuous white light.

Seeds were stored in the dark at 4 ± 1°C in a refrigerator with silica gel at least 2 months before the germination tests.

Germination Tests

Seeds were surface sterilized for 5 min in 1% (v/v) sodium hypochloride with 0.15% (v/v) Triton X-100 and rinsed with sterilized distilled water at least five times. The time when seeds were rinsed was defined as the beginning of the imbibition period in the present study. The seeds were plated in 45-mm plastic Petri plates containing aqueous agar medium or minimal salts medium (Lloyd et al., 1986). Aqueous agar medium (0.6% [w/v] agar) contained neither growth medium nor hormones. Minimal salts medium contained trace elements, vitamins, 2% (w/v) Suc, and 0.6% (w/v) agar.

Seeds sown on plates were kept in a light-tight box that was placed in a temperature-controlled dark room at 25 ± 1°C until the light treatments. After the light treatments, seeds in the Petri plates wrapped in aluminum foil were kept in the dark room until the measurements were made.

Germination frequencies were measured 5 to 7 d after the last light treatment and are expressed as the percentage of plants with protruding roots in each test population (percentage germination).

Light Treatments

White light of about 6.0 W m^{-2} from white fluorescent tubes (FL20SSW/18(G); Hitachi Ltd., Tokyo) was used for the irradiation with white light. Red light of 0.3 W m^{-2} was obtained from the same tubes as the white light but was filtered through 3-mm red acrylic (Shinkolite A102; Mitsubishi Rayon Ltd., Tokyo) and used for the red-light pulse exposure of 90 J m^{-2} by 5 min of irradiation for the induction of germination. For the far-red-light pulse exposure of 90 J m^{-2} for the inhibition of germination, far-red light of 0.3 W m^{-2} was obtained from special far-red fluorescent tubes (FL20S.FR-74; Toshiba Ltd., Tokyo) filtered through 3-mm far-red acrylic (Deraglass 102; Asahikasei Ltd., Tokyo) by 5 min of irradiation. Where necessary, seeds were manipulated under a dim green safelight (Nagatani et al., 1989).

Statistical Analysis

In all germination experiments, seeds were sown on three to five plates in lots of 50 to 100 individuals in each plate, and germination frequencies were measured after appropriate times with or without light treatments. The germination frequencies are expressed as means ± SE.

For some results, the means and SE values were compared with each other using Duncan's multiple range test (Duncan, 1955) to define whether the differences were significant. With this test, values included in a same group means that the differences among the values are not significant, but values divided into the different groups means that the differences are significant according to the unreliability (percentage) that is mentioned in each test.

Immunochemical Detection of PHYA and PHYB

Crude extracts from about 2 × 10^3 seeds were prepared and analyzed immunochemically according to the procedures described by Nagatani et al. (1993). Seeds were homogenized immediately after sterilization or incubated in the dark for 24 h on agar plates (0.8% [w/v] agar in water) before homogenization. The seeds were sterilized under white light. Homogenization was done under a dim green safelight. Etiolated seedlings grown in the dark for 5 d were used for the positive controls. The monoclonal anti-PHYA and anti-PHYB antibodies used in the present study were mAP5 (Nagatani et al., 1985) and mAT1 (López-Juez et al., 1992), respectively. Immunochemical detection of PHYA in the extracts was performed as described by Nagatani et al. (1991a).

RESULTS

PhyB Principally Regulates Germination of Seeds in the Dark and in Continuous Irradiation

Seeds of WT and the *fre1* and *hy3* mutants were plated on aqueous agar media under total darkness or continuous white light, and subsequently germination frequencies of each population were measured (Fig. 1). Under continuous white light, nearly 100% of seeds of all three genotypes germinated within 3 d (Fig. 1). In contrast, *hy3* mutant seeds did not germinate in the dark even after 15 d (Fig. 1C), whereas 60 to 70% of the WT seed population (Fig. 1A) and 50 to 60% of the *fre1* mutant seeds (Fig. 1B) germinated in the dark.

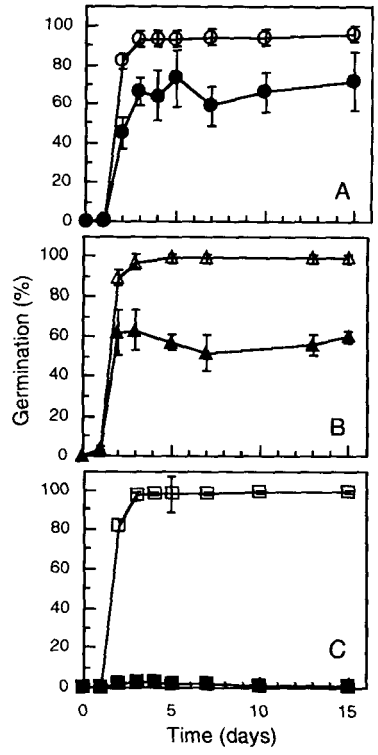

Figure 1. Time course of seed germination frequencies in WT (A), *fre1* (B), and *hy3* (C) under continuous white light and total darkness. Dormant seeds were sown on aqueous agar medium plates and allowed to imbibe for 15 d at 25 ± 1°C. The germination frequency was measured daily. ○, WT under continuous white light; ●, WT under total darkness; △, *fre1* mutant under continuous white light; ▲, *fre1* mutant under total darkness; □, *hy3* mutant under continuous white light; ■, *hy3* mutant under total darkness. Values are means ± SE from at least five determinations.

Because we harvested all seeds used in the above experiments from parents grown under the same conditions, it is likely that the *hy3* (*phy*B) mutation caused the difference in dark germination frequencies from the WT, whereas no differences in light germination under continuous white light were seen. The PhyA deficiency in the *fre1* mutant, however, showed no effect on dark germination and light germination under continuous white light. Thus, $PhyB_{fr}$ accumulated during seed production and stored in dormant seeds is likely to induce germination in the dark when seeds imbibed.

We next examined the effect of continuous irradiation with red, far-red, and white light on germination frequencies in the WT and the mutants. Continuous irradiation with white or red light fully induced germination in the *fre1* and *hy3* mutants, as well as in the WT (Table I). In contrast, the continuous irradiation with far-red light appeared to inhibit germination in the *fre1* mutant and only partially inhibited germination in the WT. This treatment significantly promoted germination in the *hy3* mutant (Table I). It is particularly interesting to note that $PhyB_r$, which was produced under continuous irradiation with far-red light, prevented the ger-

Table I. *Germination frequencies of Phy-deficient mutants under continuous red-, far-red-, and white-light irradiation*

Dormant seeds of the WT, the *fre1* mutant, and the *hy3* mutant of *A. thaliana* were sown on aqueous agar medium plates and allowed to imbibe under the different light qualities for 7 d until the measurement. Values are means ± SE from at least three determinations. The means were divided into a to c groups by Duncan's multiple range test (Duncan, 1955). D, Darkness; FR, far-red light; R, red light; W, white light.

Light Qualities	Germination (%)					
	WT		*fre1*		*hy3*	
	Mean ± SE	Duncan's test (1%)	Mean ± SE	Duncan's test (1%)	Mean ± SE	Duncan's test (1%)
D	54 ± 4	b	47 ± 6	b	2 ± 3	a
FR	14 ± 5	a	1 ± 2	a	47 ± 5	b
R	100 ± 0	c	93 ± 5	c	97 ± 3	c
W	98 ± 2	c	97 ± 3	c	98 ± 3	c

mination of *hy3* seeds, and this may have resulted from a high-irradiance reaction effect of PhyA.

$PhyB_{fr}$ in Dormant Seed Induces Germination in the Dark

In the experiments in continuous light, de novo synthesis of Phys could have been induced by imbibition or by light during the experiment. To define whether stored PhyB, de novo synthesized PhyB, or another Phy species induces germination, we examined the effect of a brief irradiation with red and/or far-red light on germination responses of the *hy3* and *fre1* mutants at the beginning of the imbibition period.

When irradiated briefly after imbibition for 1 h, red-light irradiation had a promotive effect and, when subsequently given far-red-light irradiation, photoreversibly prevented it in both the WT and the *fre1* mutant (Table II). The *hy3* mutant seeds, however, did not exhibit any responses of germination under any light treatment tested in this experiment (Table II). Since *hy3* seeds were unable to germinate in the light at the beginning of the imbibition period, this observation supports the hypothesis that PhyB is the Phy that is accumulated and stored in dormant seeds and is used to induce germination.

As shown in Table II, a brief irradiation with red light resulted in higher germination frequencies than the dark germination frequencies in seeds of the WT and the *fre1* mutant. This indicates that not only $PhyB_{fr}$ but also $PhyB_r$ is stored in dormant seeds and red-light irradiation converts $PhyB_r$ stored in dormant seeds to the $PhyB_{fr}$, which promotes the germination rate in the population.

PhyA or Other Phys Synthesized de Novo Triggers Germination in the Absence of PhyB

Because PhyA is light labile and not detected in dormant seeds (see below), a question arose as to whether PhyA synthesized during imbibition in the dark would regulate germination. Therefore, we examined the effect on light-induced germination at various times of imbibition in the dark prior to a brief irradiation with red light. The seeds of the WT and the *fre1* mutant germinated at the same levels as their white-light levels when irradiated with a brief red-light pulse regardless of the duration of imbibition periods, whereas the germination frequencies of the *hy3* mutant seeds increased to 40 to 50% depending on the duration of the imbibition in the dark (Fig. 2). It thus became evident that the *hy3* mutant seeds synthesize and accumulate some species of Phy that cannot be PhyB during an imbibition period in the dark. This observation is consistent with the observation

Table II. *Germination frequencies of Phy-deficient mutants exposed briefly to red and far-red light*

Dormant seeds of the WT, the *fre1* mutant, and the *hy3* mutant of *A. thaliana* were sown on aqueous agar medium plates, allowed to imbibe in the dark for 1 h, then exposed to red and/or far-red light (0.3 W m^{-2}) for 5 min, and kept in the dark for 4 to 6 d until the measurement. Values are means ± SE from at least five determinations. The means were divided into a to c groups by Duncan's multiple range test (Duncan, 1955). D, Darkness; FR, far-red light; R, red light.

Light Treatment	Germination (%)					
	WT		*fre1*		*hy3*	
	Mean ± SE	Duncan's test (1%)	Mean ± SE	Duncan's test (1%)	Mean ± SE	Duncan's test (1%)
D	66 ± 10	b	60 ± 3	b	1 ± 2	a
FR	3 ± 1	a	2 ± 1	a	2 ± 1	a
R	90 ± 2	c	70 ± 5	b	2 ± 2	a
R/FR	1 ± 1	a	6 ± 2	a	3 ± 1	a
R/FR/R	90 ± 1	c	71 ± 13	b	3 ± 1	a

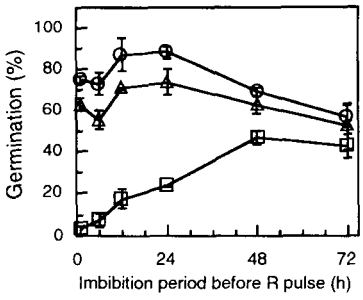

Figure 2. Effect on seed germination frequencies of duration of imbibition prior to a brief irradiation with red light (R). Dormant seeds were sown on aqueous agar medium plates, allowed to imbibe in the dark for 1 h, and then exposed to far-red light for 5 min to provide a low baseline level of germination in all three genotypes. The seeds were kept in the dark until an exposure of red light for 5 min to induce germination and then kept in the dark until the measurement. Values are means ± SE from at least three determinations. O, WT; Δ, *fre1* mutant; □, *hy3* mutant.

in the experiment of a brief irradiation with red and/or far-red light at the beginning of the imbibition period (Table II), in which previously stored PhyB regulates the light germination responses of the WT and the *fre1* mutant seeds.

Furthermore, we examined the effect of intervening darkness between red- and far-red-light irradiation on seed germination frequencies. Results of an experiment in which seeds imbibed in the dark for 1 or 24 h before the red-light pulse are shown in Figure 3. The germination frequency of the *hy3* mutant seeds was 50% if they imbibed in the dark for 24 h and the duration of time before far-red-light irradiation was longer than 6 h (Fig. 3B). In contrast, the germination frequencies of the *hy3* mutant seeds were not increased when they imbibed in the dark for 1 h before the red-light pulse and the duration of time before far-red-light irradiation was shorter than 24 h (Fig. 3A). The results also indicate that, in the absence of PhyB in the *hy3* mutant seeds, another molecular species of Phy (other than PhyB) was synthesized during the imbibition period before the red-light irradiation and regulated the light germination responses. It takes more than 1 h to synthesize a sufficient quantity of this Phy species to induce germination.

The above-mentioned data indicate that Phy(s) other than PhyB are synthesized de novo in the *hy3* mutant seeds, and this molecular species of Phy(s) plays a role in the promotion of germination by the red-light irradiation.

If the results in Figure 3B and Table I are carefully compared, it is evident that the effects of continuous irradiation and a brief irradiation of red light on *hy3* seeds are different. In the *hy3* mutant seeds, exposure to a brief irradiation with red light at the beginning of the imbibition period did not induce germination, and even after 24 h imbibition, only 30 to 50% of the seed population germinated by a brief irradiation of red light (Fig. 3B). In contrast, nearly 100% of *hy3* seeds germinated under continuous white or red light (Table I). We next tried to determine whether it was possible to substitute a repetition of pulse irradiation for continuous irradiation. We analyzed the effect of cyclically treated pulse irradiation with red light during a 24-h period on seed germination frequencies. Various numbers of red-light exposures were given after imbibition in the dark for 24 h. All of the seeds of the WT and the *fre1* mutant germinated in all light treatments, whereas the germination frequencies of the *hy3* mutant increased to 80% when the seeds were exposed to at least 24 red-light pulses at 1-h intervals (Fig. 4). In contrast, less than 30% of the *hy3* seeds germinated if fewer than 12 red-light pulses were given during 24 h (Fig. 4). This type of response seems to be regulated by molecular species of Phy(s) and requires repeated irradiations at intervals of 1 h or less to induce the germination of more than 50% of the seed population in the *hy3* mutant.

Immunochemically Detectable PHYA and PHYB in the *fre1* and the *hy3* Mutants during Imbibition

As shown in Figure 5, PHYA was detected in western blots only with the etiolated seedlings of the *hy3* mutant and the WT at approximately 116 kD (Fig. 5A, lanes 3 and 9). As already reported (Nagatani et al., 1993), no PHYA was detected in the *fre1* mutant (Fig. 5A, lanes 4–6). The sensitivity of this assay was not high enough to detect PHYA in seeds of the WT and the *hy3* mutant (data not shown). Nonetheless,

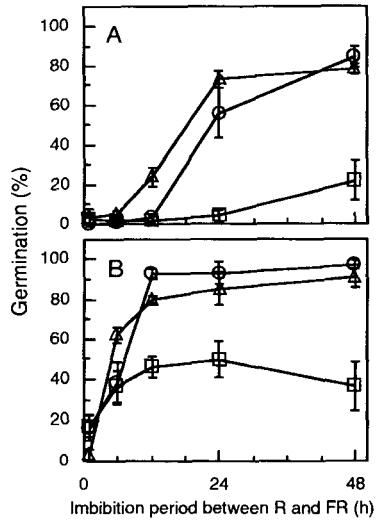

Figure 3. Effect of various intervals between brief irradiations with red light (R) and far-red light (FR) on seed germination frequencies. A, Dormant seeds were sown on aqueous agar medium plates, allowed to imbibe in the dark for 1 h, and irradiated by red light for 5 min to induce germination. After the irradiation by red light, seeds were kept in the dark during desired periods until the next exposure of far-red light for 5 min to reverse the effect of red-light irradiation. After the irradiation by far-red light, seeds were kept in the dark until the measurement. B, Dormant seeds were sown on aqueous agar medium plates, allowed to imbibe in the dark for 24 h, and then irradiated by red light. At the beginning of the imbibition period, seeds were irradiated by far-red light for 5 min to inhibit dark germination during imbibition. After the irradiation by red light, seeds were treated as in Figure 3A. Values are means ± SE from at least three determinations. O, WT; Δ, *fre1* mutant; □, *hy3* mutant.

this result indicated that amounts of PHYA in seeds were significantly lower than those in etiolated tissue in both *hy3* and the WT. In contrast, the levels of PHYB in seeds were as high as those in the etiolated seedlings in both the *fre1* mutant and the WT (Fig. 5B, lanes 4–9), suggesting that PHYB is constitutively expressed. As expected (Cone and Kendrick, 1985; Reed et al., 1993), PHYB was not detectable at all in seeds and seedlings of the *hy3* mutant (Fig. 5B, lanes 1–3). Although double bands were observed for etiolated seedlings of the WT and the *fre1* mutant, these bands are presumably due to the degradation of PHYB. For instance, when the extracts were incubated at room temperature, the density of the lower band increased and the density of upper band decreased (data not shown). The immunochemical data are consistent with the physiological conclusion that PhyB stored in dormant seed regulates seed germination.

The PhyB Signaling Pathway that Controls Seed Germination Is Separable from a DET1 Functioning Pathway

To define the relationship between PhyB and DET1 signaling pathways, we examined the effect of a brief irradiation with red and/or far-red light at the beginning of the imbibition period on germination responses of the *det1* and the *det1/hy3* mutants. When seeds were plated on minimal salts medium plates containing trace elements, vitamins, and Suc, seeds of *det1* and *det1/hy3* mutants germinated 80 to 100% regardless of the presence or absence of light (Table III). However, when seeds were plated on aqueous agar plates, the population of *det1* mutant seeds showed a red/far-red photoreversible response, but the *det1/hy3* double-mutant seeds failed to exhibit the photoreversible response (Table III) as did the *hy3* single-mutant seeds (Table II). Hence, the PhyB signaling pathway in germination may be different from the DET1 acting pathway.

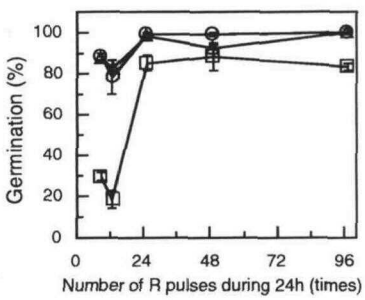

Figure 4. Effect of cyclic pulse irradiations with red light (R) on seed germination frequencies. Dormant seeds were sown on aqueous agar medium plates, allowed to imbibe in the dark for 1 h, then exposed to far-red light for 5 min, and kept in the dark for 23 h. Then they were exposed to various numbers of red-light pulses of irradiation for 5 min during 24 h and kept in the dark until the measurement. Values are means ± SE from at least three determinations. ○, WT; △, *fre1* mutant; □, *hy3* mutant.

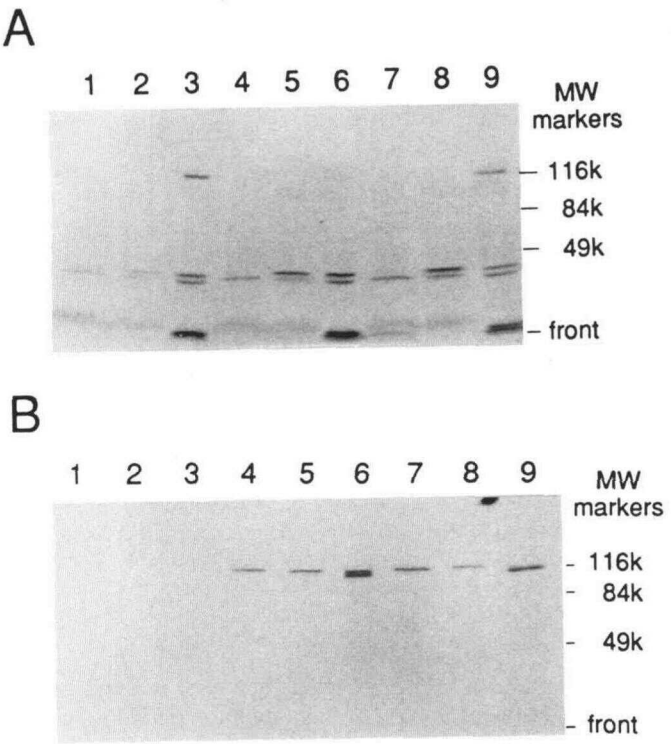

Figure 5. Immnoblot detection of PHYA and PHYB. A, Detection of PHYA in extracts from seeds and seedlings of *A. thaliana* with a monoclonal antibody mAP5. Lanes 1 to 3, *hy3* mutant; lanes 4 to 6, *fre1–1* mutant; lanes 7 to 9, WT. Lanes 1, 4, and 7, Seeds homogenized immediately after sterilization; lanes 2, 5, and 8, seeds homogenized after imbibition for 24 h in the dark; lanes 3, 6, and 9, 5-d-old etiolated seedlings. Each lane contained 100 μg of total protein, whereas 33 μg was used for the seedling. Molecular mass markers were from Sigma (Prestained Kit). B, Detection of PHYB in extracts from seeds and seedlings with a monoclonal antibody mAT1. Each lane contained samples as described for A.

DISCUSSION

Specific Function of PhyB for the Induction of Seed Germination in the Dark

Since the discovery of multiple molecular species of Phys (reviewed by Furuya, 1989, 1993), the question arises as to which Phy is associated with which of the many red/far-red photoreversible effects that have been reported in the literature since the 1950s. In the present study, we have demonstrated that PhyB principally and specifically regulates seed germination of *Arabidopsis* in the dark. Therefore, $PhyB_{fr}$ is the previously proposed stable P_{fr} form in dormant seeds (McCullough and Shropshire, 1970). PhyC, PhyD, or PhyE in the P_{fr} form, which might exist in dormant seeds, is evidently not sufficient to induce germination because *hy3* seeds did not germinate at all in the dark. In recent months, several functions have been assigned to specific molecular species of Phys using PhyA- and PhyB-null mutants to which we now add new evidence that $PhyB_{fr}$ regulates seed germination of *Arabidopsis* in the dark. EOD-FR responses have been well established as a characteristic of type II (stable)

Table III. *Germination frequencies of det1 and det1/hy3 double mutants sown on aqueous agar medium and minimal salts medium plates exposed briefly to red and far-red light*

Dormant seeds of the *det1* mutant and the *det1/hy3* double mutant of *A. thaliana* were sown on aqueous agar medium plates and minimal salts medium plates containing trace elements, vitamins, and Suc and allowed to imbibe in the dark for 1 h, then exposed to red and/or far-red light (0.3 W m^{-2}) for 5 min, and kept in the dark for 4 to 6 d until the measurement. Values are means ± SE from at least four determinations. The means were divided into a to c groups by Duncan's multiple range test (Duncan, 1955). D, Darkness; FR, far-red light; R, red light; CW, continuous white light.

Light Treatment	Germination (%)							
	Aqueous agar medium				Minimal salts medium			
	det1		*det1/hy3*		*det1*		*det1/hy3*	
	Mean ± SE	Duncan's test (1%)	Mean ± SE	Duncan's test (1%)	Mean ± SE	Duncan's test (1%)	Mean ± SE	Duncan's test (1%)
D	58 ± 6	b	12 ± 5	a	88 ± 5	a b	88 ± 6	a
FR	1 ± 1	a	8 ± 3	a	81 ± 3	a	87 ± 4	a
R	61 ± 5	b	12 ± 7	a	86 ± 5	a b	90 ± 9	a
R/FR	3 ± 2	a	7 ± 5	a	85 ± 8	a b	85 ± 1	a
R/FR/R	68 ± 1	b	7 ± 3	a	86 ± 4	a b	90 ± 4	a
CW	100 ± 0	c	93 ± 1	b	98 ± 1	b	97 ± 3	a

Phys, and the EOD-FR response is not found in PhyB-deficient mutants (Adamse et al., 1988; López-Juez et al., 1990; Nagatani et al., 1991a; Whitelam and Smith, 1991), although PhyA-deficient mutants exhibit normal EOD-FR response (Nagatani et al., 1993; Parks and Quail, 1993). Other specific responses lacking in PhyB-null mutants were the inhibition of elongation in hypocotyls, petioles, floral stems, and root hairs and accumulation of Chl (Koornneef et al., 1980; Chory, 1992; Reed et al., 1993). The PhyB-null mutants also have a reduced number of rosette leaves, and they flower earlier than the WT (Goto et al., 1991; Whitelam and Smith, 1991; Chory, 1992), whereas these responses are normal in PhyA-deficient mutants (Nagatani et al., 1993; Parks and Quail, 1993). In contrast, PhyB-null mutants have no defects in red-light induction of gene expression (Sun and Tobin, 1990; Chory, 1992). Thus, PhyB is believed to act principally in diverse tissues of seed plants throughout development and reproduction, including the regulation of seed germination under natural light conditions, whereas PhyA appears to play a role under limiting light conditions such as continuous irradiation with far-red light.

Overlapping Function of PhyA and PhyB for the Induction of Seed Germination in the Light

The present work clearly demonstrates that no Phys except PhyB are involved in dark germination. If so, do Phy(s) other than PhyB induce seed germination in the light? The finding that seeds of the PhyB-null mutants, such as *hy3* and *det1/hy3*, failed to induce the photoreversible germination in the early stage of imbibition evidently indicates that PhyB is the principal photoreceptor regulating the induction of seed germination in the light. On the other hand, when they imbibed for 24 h in the dark, seeds of the PhyB-null mutants germinated after a brief irradiation with red light (Figs. 2 and 3B). Hence, it is likely that PhyA and/or Phy(s) other than PhyB are newly synthesized and induce seed germination in the light in the absence of PhyB.

In fact, it is well known that PhyA is synthesized in seeds during imbibition in the dark (Konomi et al., 1987). Figure 5 shows that, in *Arabidopsis*, PHYB is detectable in dormant seeds at levels comparable to those found in seedlings. In contrast, PHYA is not immunochemically detectable in dormant seeds or after 24 h of imbibition in the dark, whereas it is detectable in 5-d-old etiolated seedlings. This observation suggests that PhyA is not necessary for the induction of germination or the sensitivity of the assay was not high enough to detect PHYA in seeds of the WT and the *hy3* mutant.

The WT and the *fre1* mutants showed lower germination frequencies under continuous far-red light, whereas the *hy3* mutant showed a higher frequency when compared with germination frequencies in the dark. Under these conditions, germination frequencies of *hy3* mutant seeds are also higher than the WT or *fre1* mutant (Table I). This result indicates that PhyB in seeds is converted to $PhyB_r$ by continuous irradiation with far-red light during imbibition, and thus germination is suppressed by $PhyB_r$. In the *hy3* mutant that lacks PhyB, PhyA appears to induce germination through a high-irradiance effect by continuous far-red-light irradiation. This result is in good accordance with the previous results that the inhibitory effect of continuous far-red light on hypocotyl elongation is mediated exclusively by PhyA in *Arabidopsis* seedlings through a high-irradiance effect (Nagatani et al., 1993; Parks and Quail, 1993). These data demonstrate that $PhyB_r$ plays a physiological role in seed germination, which is similar to the role for $PhyB_r$ in gravitropism in *Arabidopsis* (Liscum and Hangarter, 1993). Thus, for two independent responses, $PhyB_r$ plays an active role. This is unlike PhyA in the P_r form, which appears to be biologically inactive in all responses studied to date (Furuya, 1989).

As previously reported, under white light, PHYA-deficient mutants of *Arabidopsis* have normal length hypocotyls (Nagatani et al., 1993; Parks and Quail, 1993; Whitelam et al., 1993), but PhyB-null mutants of *Arabidopsis* (Reed et al., 1993) and PhyB-deficient mutants of cucumber (López-Juez et al., 1990) and *Brassica* (Devlin et al., 1992) have longer

hypocotyls. Thus, PhyB principally controls hypocotyl elongation under normal light condition.

However, transgenic plants overexpressing PHYA in tobacco (Keller et al., 1989; Kay et al., 1989; Nagatani et al., 1991b; McCormac et al., 1992), tomato (Boylan and Quail, 1989), and *Arabidopsis* (Cherry et al., 1992), as well as transgenic *Arabidopsis* overexpressing PHYB (Wagner et al., 1991), exhibit a short hypocotyl phenotype under similar light conditions. Therefore, if PhyA is ectopically expressed at high levels, it may play a role that is normally performed by PhyB.

Based on the present study using PhyA- and PhyB-deficient mutants, we demonstrated that Phy(s) other than PhyB are able to mediate induction of germination, which is principally regulated by PhyB in WT *A. thaliana*.

Signal Transduction Pathway from PhyB to Germination and Role of DET1

When seeds were plated on aqueous agar plates containing only distilled water in the absence of growth medium, Suc, and hormones, the *det1* mutant seeds exhibit a red/far-red photoreversible response in seed germination (Table III). In contrast, when seeds were plated on minimal salt medium containing vitamins and Suc, no light effect was observed (Table III), which is consistent with previous studies (Chory et al., 1989). Since the genetically same material was used in these studies, the difference on the germination response of *det1* mutant seeds must result from the difference in medium components. Seed germination is promoted in the presence of nitrate ions in the medium in *Arabidopsis* (T. Shinomura, unpublished data) and tomato (Georghiou and Kendrick, 1991). The *cop1* (Deng et al., 1991) and *cop9* (Wei and Deng, 1992) mutants of *Arabidopsis*, phenotypically similar to the *det1* mutant, showed Phy control of seed germination in a growth medium (Valvekens et al., 1988). The result in the present study that the *det1/hy3* double-mutant seeds fail to respond to the light treatment is consistent with the result reported previously (Chory, 1992).

Genetic studies indicated that DET1 acts downstream of both blue-light reception and phytochrome (Chory, 1992). Based on the results in Table III, DET1 may act in a distinct pathway from PhyB in the control of seed germination. However, *det1* is epistatic to *hy3* for other photomorphogenic responses, including hypocotyl growth inhibition, leaf expansion, and derepression of light-regulated gene expression (Chory, 1992). Although we did not test this directly, it is likely that COP1 and COP9 also act in distinct pathways from PhyB in the control of seed germination.

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